The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Dated 12 July 2000

Patents Form 1/77

Pate: ct 19[illegible]
(Rule [illegible])

The Patent Office

[illegible]

THE PATENT OFFICE
K
17 JUN 1999
RECEIVED BY POST

17 JUN 1999

Request for grant of a patent

(See notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

SCE/JJ/WCM.73

2. Patent application number
(The Patent Office will fill in this part)

9913979.2

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

University of Wales College of Medicine,
Heath Park,
Cardiff CF4 4XN.

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Spheroid Preparation

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Wynne-Jones, Lainé & James

Morgan Arcade Chambers
33 St Mary Street
Cardiff
CF1 2AB

Patents ADP number *(if you know it)*

1792002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
-	-	-

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*
-	-

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	None
Description	12
Claim(s)	-
Abstract	-
Drawing(s)	1 + 1

10. If you are also filing any of the following, state how many against each item.

Priority documents	-
Translations of priority documents	-
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
Request for preliminary examination and search *(Patents Form 9/77)*	-
Request for substantive examination *(Patents Form 10/77)*	-
Any other documents *(please specify)*	-

11. I/We request the grant of a patent on the basis of this application.

Signature Wynne-Jones, Lainé & James Date 16.06.99

Wynne-Jones, Lainé & James

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr. S. C. Eastwood
(01222) 229526

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Spheroid Preparation

This invention relates to a method of producing a mixture having spheroid-forming activity from fetal calf serum and two methods of spheroid formation.

Spheroids are three-dimensional cultures of cells which are normally grown in suspension. A number of processes for formation of spheroids have been proposed, for example in US 5624839, but these have been found to be relatively complex.

Spheroids in general are used in tissue culture research, for example.

According to the first aspect of the present invention, there is provided a method of producing a mixture for use in spheroid formation, the method comprising heat treatment of fetal calf serum for a time and at a temperature sufficient to impart spheroid-forming activity to the resultant mixture.

The heat treatment is preferably performed at a temperature between 60°C and 80°C, even more preferably between 65°C and 75°C. However, it is envisaged that temperatures outside these ranges could be used, particularly below these ranges, although in such a case the incubation time would be longer. The heat treatment may, for example, be performed for between 30 minutes and 7 hours. It has been found that the optimum conditions for producing the mixture are 70°C for 5 hours. However, different amounts of the mixture may be produced at different temperatures and incubation times, with generally more being

produced at the higher temperature and longer incubation time. Nevertheless, higher temperatures may give rise to too much coagulation of proteins in the serum, thus resulting in a loss of activity in the mixture.

The method may further comprise the step of storing the resultant mixture in aliquots at about -20°C.

According to a second aspect of the present invention, there is provided a mixture for use in spheroid preparation formed by the method described above.

According to a further aspect of the present invention, there is provided a method of forming a spheroid comprising contacting in a vessel a cell culture with a mixture formed by the method described above.

One or more cell types may be used, thus enabling the method to be used in the formation of heterospheroids in addition to homospheroids. Indeed, heterospheroids may be easily formed by adding several cell types in the required ratio.

The method of spheroid formation typically requires an overnight incubation period.

Spheroid size can be regulated by initial cell number, time of incubation and shape of culture vessel. Generally, small and medium sized spheroids (up to 100 micrometres), are formed after 24 hours and their size is increased thereafter mainly by fusion of spheroids rather than by cell growth.

The mixture for use in spheroid preparation may, in one embodiment be coated on to the vessel, which may be formed

of plastic. Alternatively, spheroid preparation may be carried out on uncoated vessels and, in such a case, a 5 to 10% solution of the mixture for use in spheroid preparation may be added to a medium of the cell culture.

According to a further aspect of the present invention, there is provided an elongate spheroid comprising a plurality of cells arranged linearly.

The elongate spheroids are known as "string spheroids". Typically, the elongate spheroid may have a length of at least about 1cm, or preferably about 2cm. Typically, it has been found that elongate spheroids may be of the order of 0.5mm in diameter and may typically be 25cm long, containing 100,000 - 150,000 cells per cm length. However, it should be noted that elongate spheroids may be of 100cm in length or even more.

Again, the cells may be of one or more types, thus leading two homo-or hetero-string spheroids. In one example, MCF7 and breast fibroblast cell lines have been prepared. One or more layers may be arranged around an inner elongate arrangement of cells. ECV cells have additionally been used to provide three cell layers in a triple string spheroid.

According to a further aspect of the present invention, there is provided a method of forming an elongate spheroid comprising forming a suspension by contacting a cell culture with a spheroid-forming mixture formed by the method described above at the required concentration, placing the suspension in a tubular member, incubating the contents of

the tubular member, and removing the elongate spheroid.

Typically, the required concentration is in the range of 6 to 10 million cells per millilitre. In one embodiment, the tubular member may have an internal diameter of about 1mm. Typically, the tubular member may be in the form of a "butterfly" having a length of about 25cm and an outer diameter of 2mm, but any appropriate tubing, for example one of plastic and of suitable dimensions, could be used.

The method may further comprise the step of stretching the tubular member prior to incubation, and preferably holding the tube in a horizontal position.

According to a further aspect of the present invention, there is provided a kit for forming elongate spheroids, comprising a mixture for use in spheroid formation formed by the method described above, and a tubular member.

The kit may further comprise the cells which it is desired to form into an elongate spheroid, means for placing a suspension into the tubular member, means for removing the elongate spheroid from the tubular member and/or a stand for arranging the tubular member horizontally during incubation.

Many uses for the mixture for use in spheroid formation according to this invention can be envisaged and examples include the following:

(i) It could be easily prepared as a commercially available product, either in its crude form or a purified form, for the production of homo- or heterospheroids in tissue culture research.

(ii) It could be used for the preparation of string sphe-

roids made of different cell types such as fibroblasts, smooth muscle cells, and endothelial cells to make in-vitro veins.

(iii) It could be used for the preparation of keratinocyte/fibroblast and other skin cell mini-spheroids that could be attached to an artificial support for use as a sort of skin grafting. This could produce micro-islands of skin cells on the surface of open large area wounds. The closeness of the spheroids could be controlled to give optimum outgrowth and link up of skin islands, whilst initially allowing wound exuate etc. to pass between the islands.

(iv) It could form the basis for another angle on anti-cancer therapy. When tumour cells are cultured as spheroids with the mixture of the invention, their growth is slowed right down, and the cells stick together much more strongly (hence spheroid formation). It could therefore form the basis for an anti-metastatic factor and/or an agent to slow down or even stop tumour cell growth.

Thus, according to a further aspect of the present invention, there is provided the use of a mixture for use in spheroid formation formed by the method described above in anti-cancer therapy.

For convenience, in the description below, "Spefadel" is the name given to the spheroid forming mixture of the present invention produced by heat treatment of commercially available fetal calf serum (FCS).

Although the invention has been defined above, it is to

be understood that it includes any inventive combination of the features set out above or in the following description.

The invention may be performed in various ways, and specific examples will now be described, by way of example, with reference to the accompanying drawing, in which:

Figure 1 is a diagrammatic view, partially in cross section, of a triple string spheroid formed in accordance with the invention.

Example 1 - Preparation of "Spefadel"

Heat treatment of Fetal Calf Serum (FCS) in a waterbath at a temperature of between 65 and 75°C for 30mins to 7 hours gives rise to the mixture known as 'Spefadel'. Different amounts of 'Spefadel' are produced at different temperatures and incubation times, with more 'Spefadel' being produced at the higher temperature and longer incubation time. The optimum conditions for the production of 'Spefadel' are 70°C for 5 hours. Higher temperatures, that is 75°C or above, give rise to too much coagulation of proteins in the serum, resulting in loss of 'Spefadel' activity.

No spheroid forming activity was found in FCS heat treated at 60°C for up to 4 hours, but there was 'Spefadel' activity after 7 hours incubation at this temperature.

'Spefadel' is typically prepared by heating FCS at 70°C for 5 hours and storing in aliquots at -20°C until required.

Example 2 - Spheroid Preparation with Spefadel

Spheroids can be prepared from different cell lines in ordinary sterile tissue culture flasks/petri dishes or sterile non-tissue culture flasks/petri dishes. Spheroids can be prepared in flasks/dishes that have been pre-coated with 'Spefadel' for 24hrs or longer and then washed to remove any proteins etc. that have not adsorbed to the surface. Spheroids can be prepared in flasks/dishes in the presence of 1% to 10% 'Spefadel' in any standard tissue culture medium e.g. RPMI1640, DMEM, DMEM/F12 etc. Spheroids are only formed by cells in suspension and not by cells already attached to plastic tissue culture vessels. If 10% 'Spefadel' medium is added to subconfluent monolayers of all cell types tested, they continue to grow as monolayers and grow at almost the same rate as cells cultured with FCS supplemented medium. 'Spefadel' under these conditions is completely non-toxic to the cells.

Spheroid Preparation on Coated Plastic Vessels

The vessel to be used for the preparation of spheroids can be of virtually any type of non-toxic plastic suitable for cell culture, but must be sterile. Typical vessels used successfully have included Nunc/Sterilin 25cm^2 tissue culture flasks, Sterilin 90mm bacteriological plates, Falcon 25mm and 50mm tissue culture plates, and 96, 24 and 6 well Nunc microtest plates.

'Spefadel' at about 1ml/15sq cm of plastic surface was added and spread evenly over its surface. The vessel was then placed in a 37°C incubator for between 24 and 72 hours.

After the required time the 'Spefadel' was removed and the surface of the vessel was given 3x 10min washes with 5ml aliquots of serum free medium (such as DMEM/F12) before adding about 4ml of the same medium containing 1mg/ml Bovine Serum Albumin (BSA), penicillin (100units/ml), streptomycin (100μg/ml) and fungizone (2μg/ml) (these three antibiotics together at these concentrations are known as PSF).

Breast tumour cell lines such as MCF7, MDA231 and BT474, human fibroblasts from breast and skin and an endothelial cell line such as ECV have all been used to prepare spheroids on coated plastic vessels. Basically cells were cultured as monolayers in a standard fashion in 25cm^2 Nunc tissue culture flasks with DMEM/F12 containing 10% FCS and PSF in a 37°C incubator with 5% CO_2, until almost confluent when they were made into a cell suspension with trypsin/EDTA (0.05% porcine trypsin and 0.05% EDTA in phosphate buffered saline). Cells were made up in complete 10% FCS medium and counted before centrifugation at 400G and resuspension at 1 million cells/ml in SFM with PSF and BSA. For homospheroids about 1ml of the cell suspension was added to each 25cm^2 flask and left in the CO_2 incubator for 24 hrs, after which time spheroids were formed as clusters of 20 to hundreds or even thousands of cells. Initially small spheroids were formed by attachment of cells to each other and then larger spheroids were formed by the fusion of small spheroids. Generally speaking spheroid size can be modulated by the number of cells used and the length of time they are left together. Increasing either incubation time

or cell number usually gives an increase in the size of spheroids.

Heterospheroids with different ratios of cells can easily be prepared. For example the addition of 250 000 fibroblast cells to 1 million MCF7 cells gives rise to spheroids with 4 times as many MCF7 cells as fibroblasts. The fibroblasts always end up at the centre of the heterospheroid surrounded by MCF7 cells, regardless of cell number ratios or even if the fibroblasts are added to MCF7 cells that have already formed spheroids.

Spheroid Preparation on Uncoated Plastic Vessels

The culture vessels and basic medium to be used for the preparation of spheroids on uncoated plastic are exactly the same as those used for the coated method. The main differences in the method is the addition of 5 to 10% 'Spefadel' to the basic culture medium instead of 1mg/ml BSA. All other conditions used for the preparation of spheroids on coated plastic apply to the preparation of spheroids on uncoated plastic.

Example 3 - String Spheroid Preparation

String spheroids are made from cells prepared in suspension in 10% 'Spefadel', similar to those for spheroids on uncoated plastic. In order for cells to form a complete string they have to be seeded at a certain concentration so that there are enough cells present to form a complete string but not too many cells present so as to use up all

the nutrients and give rise to excessive cell death.

Actual cell numbers used for string spheroids also depend on the cell type used and some cells such as fibroblasts only form short lengths of string spheroid, probably due to weaker connections between the cells, when compared to cells of epithelial type such as MCF7 or BT474 tumour cell lines.

Cells are prepared in suspension in 10% 'Spefadel' medium as previously described. For most cell types the optimum cell number for string spheroid preparation is between 6 and 10 million cells/ml. For MCF7 and BT474 cells the optimum is about 8 million cells/ml. Once the cells are prepared in suspension at the required concentration they are ready to be placed in a disposable sterile string spheroid apparatus. The apparatus currently used is very simple and consists of a sterile 21 gauge "butterfly" (Registered Trade Mark) with a tube length of about 25cms of internal/external diameter about 1mm and 2mm respectively. The "butterfly" is a hollow needle connected to a luer syringe connector by a hollow plastic tube. Other sizes may be used.

The method for string spheroid preparation of MCF7 cells will now be described.

Prepare a suspension of 8 million MCF7 cells/ml in 10% 'Spefadel' as already described. Take a 1ml disposable syringe and suck up 0.65ml of 10% 'Spefadel' medium and then, taking care not to get any air bubbles, suck up slowly 0.35ml of the MCF7 cell suspension, whilst holding the

syringe vertical, so that it forms a separate layer in the syringe. Connect the syringe to the butterfly and slowly press the syringe whilst still holding vertical until the suspension reaches the end of the plastic butterfly tube (care must be taken to avoid the introduction of air bubbles, as these will cause breaks in the string spheroid) which will be about 0.35ml in volume. Immediately slightly stretch the tubing over a horizontal holding frame so that the tube is held in a straight line in a horizontal position. Several string spheroids are usually made at any time and the current holding frame can accommodate up to 6 tubes. The whole process is done aseptically in a laminar flow hood to minimise contamination by microorganisms. The frame and tubes are now placed in a 5% CO_2 incubator and left overnight (18 hours). After this time the tubes are removed singly and cleaned with a steriswab before cutting the plastic tube aseptically close to the needle end of the butterfly. The tube contents are then ejected slowly (by gently pressing the syringe to push the remaining 0.7ml of medium through the tube) into 10ml of 1% 'Spefadel' medium in a 90mm sterile plastic plate. The result is a 'string spheroid' of MCF7 cells about 20cm long containing about 150,000 cells per cm length.

Hetero-string spheroids containing 2 and 3 cell types have also been prepared using the same method. Heterospheroids containing MCF7 and breast fibroblast cell lines have been prepared using cell suspensions containing 6 million MCF7 and 3 million fibroblasts per ml of medium.

In this hetero-string spheroid the fibroblasts are always at the centre surrounded by MCF7 cells. In the triple string spheroid ECV cells were also present in the cell suspension and these formed a layer of cells around the MCF7 cells to give three cell layers as shown in Figure 1.

List of Abbreviations

abbreviation	description
BSA	Bovine serum albumin
BT474	Breast tumour cell line
CO2	Carbon dioxide
DMEM	Dulbeccos Minimal Essential Medium
DMEM/F12	Dulbeccos Minimal Essential Medium/ Hams F12 tissue culture medium
ECV	Endothelial cell line
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
MCF7	Breast tumour cell line
PFS	penicillin (100u/ml), fungizone (2µg/ml) and streptomycin (100µg/ml)
RPMI1640	Roswell Park Memorial Institute 1640 tissue culture medium

FIGURE 1